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Identification of Late Differentiation Antigens of Human Cornified Epithelia, Expressed in Re-Organized Desmosomes and Bound to Cross-Linked Envelope

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Little is known about the process leading to desquamation in cornified epithelia. We describe late differentiation antigens (Ag) specific for human cornified squamous epithelia, defined by two murine monoclonal antibodies (MoAb), G36-19 and B17-21, produced after immunization with plantar stratum corneum (SC). Histologically, in epidermis both Ag are cytoplasmic in the lower stratum granulosum (SG), become pericellular in the upper SG, and progressively disappear in the lower SC. In contrast, they persist up to the desquamating corneocytes in the palmoplantar epidermis and hard palate epithelium, as well as in the three cornified epithelial components of the inner root sheath (IRS) of the hair follicle (HF). Cytologically, both Ag are expressed as surface spots only on rough corneocytes. They are largely preserved on cross-linked envelopes (CLE) of the fragile type. Ultrastructurally, both Ag appear in keratinosome-like cytoplasmic vesicles in the upper stratum spinosum (SS) and the SG keratinocytes, then are found in both the regular and reorganizing desmosomes of the SG keratinocytes, and lastly in the corneocyte-specific reorganized desmosomes we propose to name corneodesmosomes. On CLE, the Ag are located on fibrils gathered over the external side of the envelope. Immunochemically, the G36-19–defined epitope is sequential and shared by five non-cytokeratin protein antigens of molecular weight 33.5, 36.5, 40, 49, and 52 kD, the higher molecular weight polypeptides being possibly precursors of the 33.5-kD protein. In contrast, the B17-21 epitope, unaccessible by immunoblotting, is probably conformational.

In long-term cultured keratinocytes, the Ag are only expressed when epidermal sheets are morphologically differentiated. The expression is enhanced in the absence of fetal calf serum (FCS) and of epidermal growth factor (EGF).

G36-19 and B17-21 Ag participate in a corneodesmosome-CLE superstructure that is probably involved in corneocyte cohesiveness and partly responsible for the mechanical resistance of the SC. These Ag are relevant markers for studying desmosomal maturation during epidermal differentiation and desquamation. *J Invest Dermatol 97:1061–1072, 1991*

The terminal differentiation of stratified squamous epithelia is characterized by a series of morphologic and metabolic changes that starts when a keratinocyte leaves the germinative basal layer, continues along the SS, and finally produces a flattened cell that exfoliates from the surface of the epithelium. The differentiation program that controls these changes varies with anatomical site. In human epidermis, hard palate, and gingival epithelia, it induces in the latest stages the formation first of a SG then of a SC horny layer comprised of corneocytes. In the course of their differentiation program, the keratinocytes of cornified epithelia synthesize various proteins such as cytokeratins, some of them being specific [1–3], profilaggrin

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Abbreviations:

Ab: antibody/ies
Ag: antigen(s)
2ME: 2-mercaptoethanol
CLE: cross-linked envelope
EGF: epidermal growth factor
FCS: fetal calf serum
HF: hair follicle
IIF: indirect immunofluorescence
IRS: inner root sheath
MoAb: monoclonal antibody(ies)
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
SC: stratum corneum
SDS: sodium dodecyl sulfate
SG: stratum granulosum
SS: stratum spinosum
TBS: Tris-buffered saline

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[4,5], involucrin, keratolinin, and loricrin [6–9], which participate in the formation of specific structural components such as the cytoplasmic intermediate filament network [10], keratohyalin granules [11,12], and CLE [13], respectively. In the upper layers of the SG, the keratohyalin granules are dispersed and the histidine-rich profilaggrin is proteolyzed into many basic filaggrin subunits that interact with cytokeratin polypeptides linked to each other by disulfide bonds, and could contribute to their aggregation into highly insoluble macrofibrils [14]. At the same time, CLE is elaborated beneath the plasma membrane by a specific transglutaminase that catalyses the Γ-glutamyl-ϵ-lysine isopeptide cross-linking of precursor proteins. In normal epidermis, the CLE shows a crumpled surface in lower SC (fragile CLE); then it smoothes out and becomes totally hardened in upper SC (rigid CLE) [15]. In mid-SC, the filaggrin molecules become more acidic, then dissociate from the cytokeratins [14], are extensively degraded by endogenous proteases, and provide amino acids that could contribute to the maintenance of SC hydration [16]. During the late stages of epidermal differentiation changes also occur in cytokeratins because the 68-kD and 56–56.5-kD cytokeratins (numbers 1 and 10–11 of the Moll catalogue [17]) undergo proteolysis that generates the 65-kD to 63- and 55-kD cytokeratins typical for the SC [10,18]. These various aspects of the cornifying differentiation program can vary with the anatomical site but also be physiologically modulated and dyssynchronized in pathology.

In contrast to these cytoplasmic events, little is known about the evolution of the keratinocyte plasma membrane during cornification or about the mechanisms that are involved in the SC cohesiveness. During the cornification process, the plasma membrane was shown to be replaced by a monolayer of N-Ω-hydroxyacyl sphingosines chemically bound to the CLE, probably by ester bonds with involucrin [19]. In the upper part of the SG, the membrane-coating granules or keratinosomes [11,20] extrude their lipid content into the intercellular space [21]. This material, which essentially contains ceramides, cholesterol, and free fatty acids, is believed to undergo biochemical and biophysical changes to form the SC intercellular lipid lamellae [22]. Therefore, the SC has been compared to a brick wall where corneocytes are embedded in a lipid-rich intercellular cement that contributes to the epidermal permeability barrier. Furthermore and particularly since the description of steroid sulfatase deficiency in X-linked ichthyosis [23], which impairs lamella dissociation, the intercellular cohesiveness has essentially been attributed to this lipid-rich matrix [24]. This hypothesis was supported by the description of drastic structural modifications of desmosomes occurring in SC, which were considered as a desmosome degradation [25–27]. However, corneocyte cohesion was recently found to be sensitive to proteases and thus, at least partly, to be due to protein bonds [28–31]. Moreover, the delayed desmosomal disappearance that has been described in several desquamation disorders such as ichthyoses and psoriasis and recent ultrastructural arguments [32,33] led us to consider that, although modified, the SC desmosomes contribute to corneocyte cohesiveness and are involved in the regulation of SC desquamation.

In this study, we present two murine MoAb, G36-19 and B17-21, specific for components of the modified desmosomes of cornifying cells we propose to call "corneodesmosomes." We describe the immunocytohistochemical localization by light and electron microscopy, and the biochemical characteristics of the Ag they define. The relationship of the corneodesmosome Ag with the CLE as well as their expression in cultured keratinocytes is also detailed.

MATERIALS AND METHODS

Chemicals and Immunologic Reagents All chemicals used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting were purchased from Bio-Rad Laboratories (Richmond, CA). Nonidet P-40 (NP-40), phenylmethylsulfonyl fluoride, ethylenediaminetetraacetate (EDTA), N-ethylmaleimide, aprotinine, and proteinase K, were obtained from Sigma Chemical Co. (St Louis, MO), and buffer salts from E. Merck Co. (Darmstadt, FRG). Tissue-tek medium was obtained from Reichert-Jung (Heidelberg, FRG) and Lowicryl K4M from Chemiche Werke Lowi (Waldkraiburg, FRG). KL1 and BL6 MoAb were purchased from Immunotech (Luminy-Marseille, France), fluorescein isothyocyanate (FITC)-labeled rabbit Ig and peroxidase-conjugated sheep Fab fragments, specific for mouse IgG (H + L), from Biosys (Compiègne, France), and GAM goat IgG G-10 colloidal gold-conjugated (10-nm gold granules), specific for mouse IgG from Janssen Life Sciences (Beerse, Belgium). All culture chemicals including culture media, antibiotics, epidermal growth factor (EGF), and fetal calf serum (FCS) were obtained from Serva (Heidelberg, FRG).

Tissues, Cells, and Cell-Lines All the tissues and cells used were of human origin. Normal skin from various anatomical sites was obtained from patients undergoing amputation or plastic surgery (foot, leg, thigh, scalp, breast, arm, abdomen). Mucosae with non-cornified squamous epithelia (vagina, uterine cervix, esophagus, and cornea) and with other types of epithelia (e.g., bladder, trachea) came from necropsies. Hard palate was obtained from patients undergoing surgery. Corneocytes were collected by scraping hard palate and epidermis of leg, arm, and foot sole. The A431 cell line was kindly provided by C. Etievant (Laboratoire Pierre Fabre, Castres, France), the HT29 cell line by J.-C. Murat (Institut de Physiologie, Toulouse, France), and the Detroit 562 cell line by W.W. Franke (Cancer Research Institute, Heidelberg, FRG).

Monoclonal Antibody Production Human plantar SC scraped from four healthy volunteers was finely crushed in 0.15 M NaCl, 7 mM K_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, phosphate-buffered saline (PBS) and the homogenate was used as immunogen. Aliquots (200 μl) were injected intraperitoneally in the presence of Freund's complete adjuvant to BALB/c mice. Injections were repeated after 2 and 4 weeks, using Freund's incomplete adjuvant in the third injection. Mouse sera were assayed for anti-cytokeratin antibodies (Ab) by a specific ELISA and for anti-epidermal Ab by indirect immunofluorescence (IIF) on cryosections of normal skin (see below). The mouse with the highest level of circulating Ab was boosted (fourth injection) and sacrificed 3 d later. Hybridomas were produced, according to Kölher and Milstein [34], by fusing splenocytes with mouse myeloma SP 2O–Ag 14 cells, in 40% polyethyleneglycol 4000, for 1.5 min at 37°C. Hybridoma supernatants were screened by the same techniques as mouse sera. The hybridomas that secreted Ab reactive in one or both tests were twice cloned, then intraperitoneally injected to BALB/c mice. From 10 d to 2 weeks later, ascitic fluids were withdrawn and the MoAb were purified by ammonium sulfate precipitation, dialysis, then chromatography on DEAE Trisacryl LS (IBF, Villeneuve la Garenne, France). MoAb isotypes were determined by MonoAb-ID ELISA (Tago, Burlingame, CA) and verified by Diffugen radial immunodiffusion (Tago, Burlingame, CA).

Enzyme-Linked Immunosorbent Assays A previously described indirect ELISA [35], using human plantar SC cytokeratin polypeptides solubilized in 8 M urea, 1% 2-mercaptoethanol (2ME), 100 mM Tris, pH 7.4, as immunosorbent, was used to detect anti-cytokeratin Ab. Cross-reactivities with other tissue protein Ag were investigated by indirect ELISA using purified tubulin, actin, myosin, tropomyosin, spectrin, transferrin, thyroglobulin, and myoglobin (generous gifts from S. Avrameas, Institut Pasteur, Paris, France) solubilized in 0.1 M $NaHCO_3$-Na_2CO_3 buffer, pH 9.5, as immunosorbent, according to Guilbert, Dighiero, and Avrameas [36].

Histology and Cytology (Morphology and Immunofluorescence) Histologic studies were performed on sections obtained from skin, mucosae, and sheets of stratified cultured keratinocytes. Tissue samples were either frozen-embedded in Tissue-tek medium at −80°C, or paraffin-embedded after fixation for 1 to 2 d either in Bouin's solution or in absolute ethanol. The frozen-embedded tissues were cut at −30°C into 4 μm-thick cryosections and the sections were air-dried. The paraffin-embedded tissues were cut into 4-μm sections, then deparaffinized in a graded series of ethanol/PBS just before use.

Cytologic analyses were performed on corneocytes scraped from various sites, on plantar corneocytes previously treated with a buffer containing NP-40 (see below), and on plantar CLE. All these cytologic materials were suspended in 0.02% sodium azide PBS at a concentration of 300 cells/mm^3, then cytocentrifuged at 700 rpm (Shandon Cytospin, Cheshire, GB) for 15 min on 0.5% gelatin-coated slides and air-dried. Cytomorphologic studies were performed by examination under phase contrast or differential interference contrast microscopy and enumerations were performed in quadruplicate on corneocytes and CLE coming from two or more scrapings.

For IIF, the sections and cytocentrifuged cells were first hydrated for 15 min in PBS, then incubated with MoAb diluted in PBS, or with PBS alone as a negative control, for 1 h at 37°C in a moist chamber. The purified G36-19 MoAb was used at concentrations ranging from 5 to 10 μg/ml and the B17-21 ascitic fluid was diluted to 1:50 in PBS. The slides were washed twice for 7 min, in 0.05% Tween 20 PBS and PBS successively, then incubated with FITC-labeled rabbit Ab to mouse IgG (H + L) diluted to 1:50 in PBS for 15 min. After a rinsing sequence as above, the slides were prepared with Fluoprep medium (Biomérieux, Lyon, France) and observed under a BH2 microscope with UV epiillumination (Olympus, Tokyo, Japan). Photographs were taken with an OM4 camera (Olympus, Tokyo, Japan) using Kodak Ektachrome 160 ASA films.

Cross-Linked Envelope Preparation Plantar CLE were purified using different methods. Equal amounts of plantar corneocytes were extracted either 2 or 4 times in 10 mM Tris-HCl buffer, pH 7.5, containing 5% SDS and 2% 2ME. In both cases, each extraction step was performed either by boiling for 10 min under stirring, or by stirring for 2 h at room temperature. Pellets were recovered by centrifugation at 15,000 × *g* for 30 min, rinsed twice in large volumes of 0.02% sodium azide 10 mM Tris-HCl, pH 7.5, then dialyzed against 0.02% sodium azide PBS and used for cytologic and ultrastructural analyses.

Immunoelectron Microscopy

Post-Embedding Indirect Immunogold Labeling: Fresh abdominal skin fragments 4 mm wide were promptly placed in 2% paraformaldehyde PBS at 4°C, and sliced into 1 mm-thick vertical sections. Following 2 h fixation, the skin specimens were washed in PBS for 2 h at 4°C, dehydrated in a graded series of ethanol (1 h incubation steps in 30% ethanol at 0°C, 50% ethanol at −20°C, 70% and 2 times 100% ethanol at −35°C), and impregnated with Lowicryl K4M. Two successive 1-h incubations with ethanol-Lowicryl mixtures in the ratio of 1:1 and 1:2, respectively, were followed by a 1-h and then an overnight incubation in the pure Lowicryl K4M, still at −35°C. The tissue samples were then transferred to fresh Lowicryl in gelatin capsules and exposed to UV light (365 nm) for polymerization (24 h at −35°C and 3 d more at room temperature). Ultrathin sections collected on collodion-carbon coated nickel grids were used for immunocytochemical studies. The grids with the attached skin sections were first floated for 10 min on drops of PBS containing 0.1% heat-inactivated FCS, and then incubated with the G36-19 MoAb used at 2.5 μg/ml and the B17-21 MoAb diluted to 1:50, at 37°C for 45 min in a humid chamber. KL1 and BL6 MoAb, which constituted positive and negative controls, respectively, were used in parallel. KL1 was demonstrated to react specifically with several cytokeratin polypeptides and mainly with the acidic (type I) 56.5-kD cytokeratin polypeptide of human epidermis (number 10 of the Moll catalogue) [17] and to be suitable for immunogold labeling of Lowicryl K4M-embedded skin [37-39]. BL6 was shown to recognize the CD1a human cortical thymocyte Ag and to be specific for a 49-kD cell membrane protein exclusively expressed by Langerhans cells in the epidermis [40]. It was always found to be unreactive on paraformaldehyde-fixed Lowicryl K4M-embedded skin [39]. Additional control sections, non-incubated with a primary Ab, were also included. After washing in PBS, the sections were incubated with the GAM IgG G-10 immunogold conjugate (1:3 dilution) at 37°C for 45 min. Following this last incubation, the grids were washed again in PBS (3 times) and in distilled water, air dried, and stained with uranyl acetate for 5 min and lead citrate for 1 min.

Pre-Embedding Indirect Immunogold Labeling: Plantar CLE purified by four-cycle extraction at room temperature as described above were fixed in 3% paraformaldehyde PBS solution for 13 h at 4°C, washed in PBS, and incubated with primary MoAb (the same as for the Lowicryl-embedded tissues) for 30 min at 37°C. After two 10-min washes, the CLE suspensions were labeled with the GAM IgG G-10 immunogold conjugate, washed again for 30 min, postfixed in 1% osmium tetroxide, and routinely processed for Epon-embedding and standard electron microscopy observation.

Protein Extraction, Electrophoretic Analyses Proteins were extracted from scraped plantar corneocytes, from cleaved abdominal and breast epidermis, and from tape-stripped and then cleaved breast epidermis. Tape stripping [41] was repeated on thawed skin fragments until complete removal of the SC was seen on control cryosections. Epidermo-dermal cleavage was performed by heat treatment according to Dale et al [42]. Epidermis or plantar corneocytes were directly crushed in a Potter homogeneizer either in 10 mM Tris-HCl, pH 7.4, containing 2% SDS, 1% 2ME, and 10% glycerol [43] or in 40 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% NP-40, 0.1% sodium azide, 2 μg/ml aprotinine, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mM N-ethylmaleimide [NP-40 Tris-buffered saline (TBS)] [44]. After sonication (Bioblock, Illkirch, France) for 1 min at setting 30, extraction was performed overnight under gentle stirring at 4°C. Then, the samples were centrifuged for 10 min at 15,000 × *g* and the supernatants were used for SDS/PAGE on 10% acrylamide slab gels or 5-20% acrylamide gradient gels, according to Laemmli [43]. Extensive proteolysis of samples of the epidermal NP-40-TBS extracts was performed with 500 μg/ml proteinase K in the presence of 1% SDS at 37°C for 1 h; then the samples were supplemented to 2% SDS 1% 2ME and separated by SDS/PAGE. A control sample without proteinase K was tested in the same experimental conditions.

Cytokeratins were specifically extracted from epidermis of various sites including the sole, from epithelia (corneal, hard palate, uterine cervix, and vaginal epithelia) and from cultured cells (A431, HeLa, Detroit 562, HT29) according to Achstätter, Hatzfeld, Quinlan et al [45]. Two-dimensional gel electrophoresis was performed, using non-equilibrium pH gradient electrophoresis in the first dimension, as described by O'Farrell, Goodman, and O'Farrell [46] and modified by Franke et al [47]. In the second dimension, SDS-PAGE on 10% acrylamide gel was performed according to Laemmli [43]. The gels were stained with Coomassie brillant blue R250.

Immunoblotting After electrophoretic separation, the proteins were transferred to 0.22 μm pore size nitrocellulose sheets according to Towbin, Staehelin, and Gordon [48]. The sheets were pre-incubated for 30 min in 0.05% Tween 20 PBS, then with the MoAb diluted in the same solution for 1 h at 37°C plus overnight at 4°C. After washings in 0.05% Tween 20 PBS (3 × 10 min) and PBS (1 × 10 min), the sheets were incubated for 2 h at room temperature with peroxidase-labeled sheep Fab fragments to mouse IgG(H + L) diluted to 1:50 in 0.05% Tween 20 PBS. After a rinsing sequence as above, the sheets were incubated for 10 min in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5, and the reaction was developed with 2.8 mM 4-chloro 1-naphthol, 0.015% hydrogen peroxide in the same buffer. The MoAb EE21-06 (IgG1k) specific for human cytokeratins numbers 1, 2, 9, 10-11 [49] of the Moll catalogue [17] was used as positive control.

Keratinocyte Cultures During long-term cultures in the conditions described by Rheinwald and Green [50], human keratinocytes stratify and generally constitute a poorly differentiated multilayered epidermal sheet. Rarely, perhaps owing to a particular donor-speci-

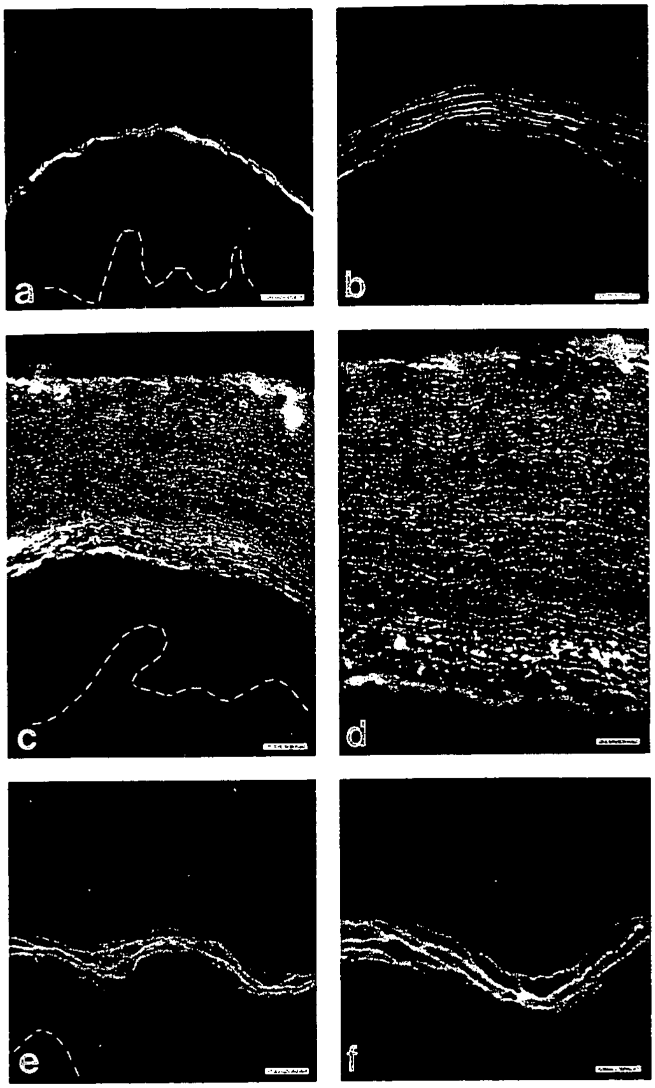


Figure 1. Indirect immunofluorescence labeling of cryosections of breast skin *(a)*, hard palate mucosa *(b)*, plantar skin *(c,d)*, and paraffin-embedded sections of plantar skin *(e,f)* with G36-19 MoAb. In breast epidermis *(a)* the labeling is restricted to the stratum granulosum and shows, in the lower keratinocytes, a cytoplasmic diffuse microgranular pattern that becomes pericellular in the upper cells then disappears in the lower stratum corneum. In hard palate epithelium *(b)* the labeling appears in the stratum granulosum and persists throughout the whole stratum corneum, where it shows a pericorneocyte microgranular pattern. In plantar epidermis *(c,d,e,f)* the labeling varies with the fixation method. On cryosections *(c,d)* the labeling appears in the stratum granulosum and persists throughout the stratum corneum up to the desquamating layer; it shows a discontinuous pericorneocyte microgranular pattern. On paraffin-embedded sections *(e,f)* the labeling is restricted to the stratum granulosum. In the lower part, the pattern is cytoplasmic diffuse microgranular, it becomes pericellular discontinuous microgranular in its upper part, and disappears in the lower stratum corneum. *Bars;* 25 μm in *a, d, e;* 16 μm in *b, f;* 50 μm in *c.*

fic ability, keratinocytes stratify and produce a morphologically fully differentiated epidermis with a SG and a SC (personal observation). Moreover, in both cases, the differentiation state of the cultures can be modulated by the composition of the culture medium. The expression of the Ag defined by G36-19 and B17-21 was analyzed for both cases and with either classical culture media or media able to induce keratinocyte differentiation. Normal human keratinocytes from abdominal skin of two subjects were cultured as follows. After storage overnight in Hanks' solution lacking Ca^{++} and Mg^{++} and containing antibiotics (400 μg/ml streptomycin, 400 units/ml penicillin, 1 μg/ml fungizone), skin slices were prepared with a keratome and epidermis was separated from dermis by incubation in a Puck's saline solution 0.25% trypsin and 0.02% EDTA for 1 h at 37°C. Epidermis was removed with fine forceps and placed in the same Hanks' solution as above containing 10% FCS. A cell suspension was prepared by repeated pipetting and agitation of the epidermal sheets. Primary cultures were grown on irradiated 3T3 feeder layers. Subcultures were obtained by seeding culture dishes at 2×10^4 cells/cm^2 on 3T3 cells. The cells were grown at 37°C in 5% CO_2 in the same medium renewed every 48 h, for 3 weeks. Four different culture media were used: (A) standard medium, i.e., ¾ minimum Eagle's medium and ¼ Ham's medium, supplemented with 10% FCS, 0.5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 5 μg/ml insulin, 0.2 mg/ml adenine, 2.6 μg/ml transferrine, 0.14 μg/ml triiodotyronine, and 0.17 μg/ml EGF; (B) standard conditions with delipidized versus normal FCS; (C) standard conditions without FCS; (D) standard conditions without EGF. After 3 weeks, keratinocyte sheets were recovered by scraping and used for morphological and IIF studies.

RESULTS

Monoclonal Antibodies Six hundred twenty-five hybridoma supernatants were screened: 112 were shown, by IIF, to contain Ab specific for various antigenic structures of the skin. Among them, 18 were shown, by ELISA, to be specific for epidermal cytokeratins. Fifty-six of the 112 selected hybridoma populations that survived and maintained their Ab secretion, were stored in liquid nitrogen. Twenty of the 56 hybridoma supernatants were shown to react with several of the various non-cytokeratin proteins assayed by ELISA and suspected to contain polyreactive Ab; they were not further studied. The remaining 36 hybridoma populations were cloned twice and the related MoAb were produced as mouse ascitic fluids. Among the MoAb unreactive to epidermal cytokeratins and showing histologic specificities towards skin components, G36-19 (IgG1k) and B17-21 (IgG1k), which specifically labeled the SG in epidermis and the inner root sheath (IRS) in HF, were further characterized.

Morphologic Characterization

Histology: When histochemically screened on various human tissues by IIF, G36-19 and B17-21 were found to be unreactive with all the non-epithelial tissues and with most of the epithelia, including non-cornified squamous epithelia. In contrast, they labeled epidermis and hard palate epithelium, as well as the IRS of the anagen HF. In almost all of the anatomical sites the labeling of epidermis was found to be mainly restricted to the SG (Fig 1*a*). The lower SG keratinocytes showed a cytoplasmic diffuse microgranular labeling that became closely pericellular in the uppermost SG keratinocytes, then progressively decreased and disappeared in the lower part of the SC. On hard palate epithelium, G36-19 and B17-21 labeled all the cell layers from the SG to the upper SC (Fig 1*b*). These patterns of labeling were found on cryosections as well as on paraffin-embedded tissues. On plantar epidermis cryosections, the SG was labeled in the same way but pericellular labeling persisted throughout the whole SC including the uppermost layers (Fig 1*c, d*). In contrast, on plantar epidermis paraffin-embedded sections only the SG labeling persisted (Fig 1*e, f*).

Within the HF, G36-19 and B17-21 exclusively labeled the IRS (Fig 2). The labeling was found to appear asynchronously in the three compartments of the IRS, first in the Henle layer, then in the cuticle, and lastly in the Huxley layer (Fig 2*a*). Like in the epidermal SG, the deepest positive IRS cells showed a cytoplasmic microgranular labeling (Fig 2*d*) that rapidly became pericellular in the more differentiated cells (Fig 2*e, f*). This labeling persisted all along the IRS (Fig 2*a–c*) up to its exfoliating area, near the isthmus of the sebaceous gland. The reactivity to IRS was found to be preserved on paraffin-embedded sections.

Cytology:

Corneocyte morphology Among scraped corneocytes, phase-contrast microscopy allowed the two previously described cell types [51,52], i.e., corneocytes with a rough surface and an irregular shape and corneocytes with a smooth surface and a regular polygonal outline, to be distinguished and counted. Their proportions varied according to the anatomical site of scraping: 90% of the corneocytes from foot sole were "rough" cells, whereas about 80% of the corneocytes from leg and arm were "smooth" cells.

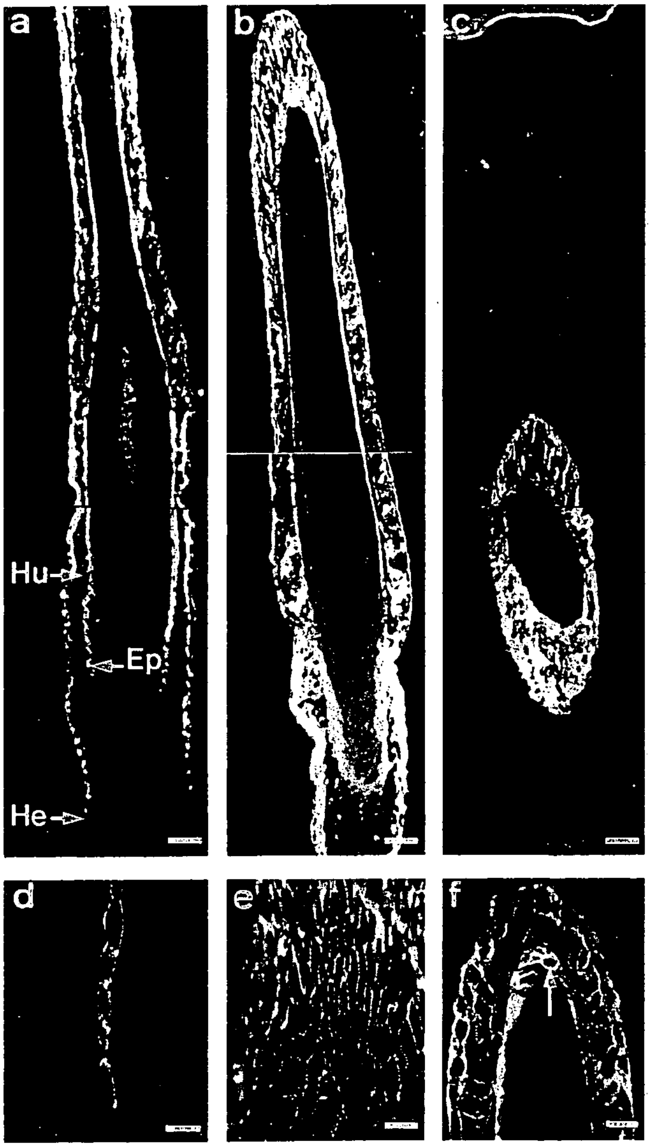


Figure 2. Indirect immunofluorescence labeling of cryosections of scalp anagen hair follicle with B17-21 *(a,b,c)* and G36-19 *(d,e,f)* MoAb. On serial longitudinal sections of hair follicle *(a,b,c)* the labeling is restricted to the inner rooth sheath (IRS). In the bulb of the hair follicle, the labeling appears first in the Henle layer (He), then in the cuticle of the IRS, i.e., the epidermicle (Ep), and lastly in the Huxley layer (Hu). It persists in the three layers all along the IRS up to the isthmus. In the bulb, the lowest cells of the Henle layer *(d)* show microgranular cytoplasmic labeling that becomes closely pericellular higher in the layer. Tangential section of the mature IRS *(e)* shows a net-like pattern due to pericellular labeling. Cross section of the mature IRS *(f)* shows a pericellular microgranular labeling in the three layers. Note the dusty membrane-associated labeling of cells of the cuticle of the IRS *(arrow)*. *Bars:* 33 μm in *a, b, c;* 15 μm in *d, e, f.*

Corneocyte immunofluorescence IIF with G36-19 and B17-21 showed that the immunoreactivity of corneocytes also varied according to their anatomical origin. Indeed, 90% of the corneocytes from foot sole and from hard palate (Fig 3) were strongly labeled, whereas only 10 to 20% of the corneocytes from leg and arm were reactive and then only with a weak labeling intensity. However, whatever their origin, the corneocytes showed the same labeling pattern, i.e., punctate, cell-membrane-associated labeling with a regular and homogeneous distribution of fluorescent desmosome-sized dots. On hard palate corneocytes (Fig 3) and on plantar corneocytes, unreactive lines, appearing as ribs on the cell surface, separated the labeled polygonal areas. The concordance between the percentages of "rough" corneocytes and labeled corneocytes, whatever the site of scraping, suggested that only this type of corneocyte expressed the studied Ag. Double examination by phase-contrast microscopy and IIF confirmed this correlation between corneocyte shape and immunoreactivity.

Cross-linked envelope morphology Differential interference contrast microscopy analysis of CLE prepared from plantar corneocytes allowed the fragile and rigid CLE to be distinguished (Fig 4*a*) and counted [15]. They constituted about 90% and 10% of total CLE, respectively.

Cross-linked envelope immunofluorescence On plantar corneocyte CLE, the reactivity to G36-19 and B17-21 was found to be preserved and the labeling pattern was closely similar to the pattern observed on the intact plantar corneocytes (Fig 4*b*). However, the

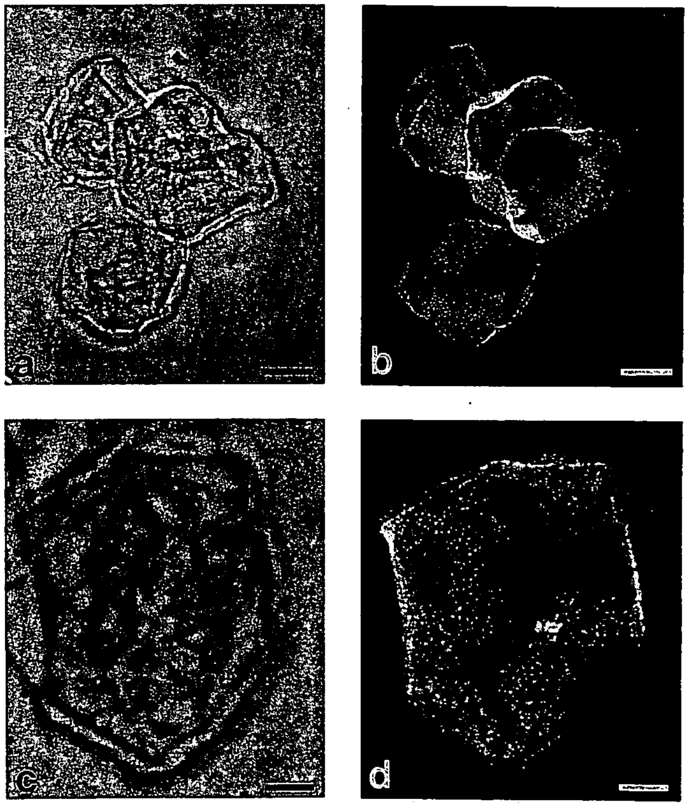


Figure 3. Double examination by phase-contrast microscopy *(a,c)* and by immunofluorescence with G36-19 MoAb *(b,d)* of corneocytes scraped from hard palate. The corneocytes show an irregular polygonal outline; their surface is rough, cut up by small valleys *(a,c)*. They show dotted labeling regularly and homogeneously distributed over their surface; unreactive lines separate polygonal labeled areas *(b,d)*. *Bars:* 20 μm in *a, b;* 8 μm in *c, d.*

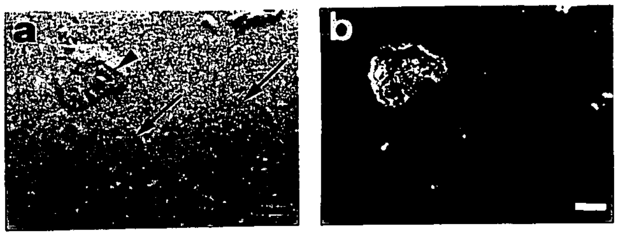


Figure 4. Double examination by differential interference contrast microscopy *(a)* and by immunofluorescence with G36-19 MoAb *(b)* of fragile and rigid plantar cross-linked envelopes. Differential interference contrast microscopy shows fragile *(arrowhead)* and rigid *(arrow)* cross-linked envelopes. Immunofluorescence shows that only the fragile cross-linked envelope is labeled, whereas the two rigid envelopes are entirely negative. *Bar,* 15 μm.

percentage of labeled CLE varied according to the thoroughness of the purification method. Thus, about 90% of CLE were immunoreactive after the extractions performed at room temperature versus 50% after the two-step and 30% after the four-step extractions performed under boiling. Double examination by differential interference contrast microscopy and IIF showed that the immunoreactive CLE were exclusively of the fragile type (Fig 4).

Ultrastructure:

Immunogold labeling of epidermis The electron microscopy study of epidermis using immunogold labeling with G36-19 and B17-21 showed that the two MoAb had the same reactivity pattern, though G36-19 gave stronger labeling. Both the MoAb detected Ag localized within the junctional structures between the keratinocytes of the most superficial SG and between corneocytes in the SC (Fig 5). The Ag first appeared in the outermost keratinocytes of the SS. In these cells, the gold granules were exclusively localized in small cytoplasmic vesicles (100–200 nm) bearing traces of a fine lamellar structure (Fig 5*d, e*). Desmosomes of the SS and lower SG keratinocytes were unlabeled. The intracellular labeling persisted up to the most superficial SG cells, where the Ag also became detectable in the well-structured desmosomes (Fig 5*d*). Further out, at the interface between the SG keratinocytes and the corneocytes, as well as in the SC itself, the labeling was observed selectively in the desmosome-derived structures, the corneodesmosomes (Fig 5*a–c*). The immunogold marker was predominantly localized in the junctional intercellular substance, which at this stage of differentiation became amorphous. In the SC, cytoplasmic desmosome plates with the attached cytokeratin filament bundles disappeared, and the homogeneous CLE outlining the corneocytes became clearly visible. An electron-lucid layer was distinguished at the surface of the corneocytes. It was particularly well visualized at places of cell-to-cell attachment.

The labeling was the strongest in the lower SC. More superficially, both the frequency of the corneodesmosome intercellular discs and the intensity of their immunogold labeling decreased. However, the G36-19 and B17-21 Ag, although rare, were present up to the last cells remaining attached to the SC (in our specimens: 9 to 11 cornified keratinocyte layers). The controls revealed no specific immunoreactivity of the BL6 MoAb on the paraformaldehyde-fixed skin, but only a weak background labeling that was roughly comparable to the labeling obtained with immunogold conjugate alone. In contrast, specific immunogold labeling was obtained with the KL1 MoAb (Fig 5*f*), which labeled cytoplasmic cytokeratin filaments in all the suprabasal layers including the SC. None of the control MoAb labeled the extracellular parts of the desmosomes or corneodesmosomes.

Immunogold labeling of cross-linked envelopes On CLE, G36-19 (Fig 6*a*) and B17-21 (Fig 6*b*) immunogold labeling confirmed the IIF observations indicating the persistence and uneven distribution of the Ag. The Ag were present exclusively on the extracellular sides of the structures, clearly distinguishable from the intracellular fuzzy features by their smoother appearance. The labeled spots were periodically distributed along the CLE and contributed to the clearer visualization of the lucent superficial band representing the lipid portion of the envelope [19]. There was no qualitative difference between the results obtained with G36-19 or B17-21 but the latter gave somewhat weaker labeling. The portions of CLE expressing the G36-19 and B17-21 Ag appeared to be more rigid than the unlabeled parts. The BL6 control MoAb gave no labeling of the CLE. The KL1 MoAb recognized cytokeratins in filaments present on the fuzzy side of the structures.

Immunochemical Characterization

NP-40–TBS Soluble Extracts from Epidermis and Plantar SC (Fig 7a): Within the extracts from whole abdominal and breast epidermis, G36-19 labeled after immunoblotting a set of five Ag of 33.5, 36.5, 40, 49, and 52 kD, the latter Ag being more strongly labeled. On proteins from tape-stripped breast epidermis it labeled the same set of Ag but the reactivity to the 33.5-kD Ag was almost absent. The immunoreactivity entirely disappeared when these extracts were previously digested by proteinase K. On extracts from plantar SC, G36-19 showed no immunoreactivity.

SDS-2ME Soluble Extracts from Plantar SC (Fig. 7b): Because of the predominance of cytokeratins in the extracts, the gels had to be overloaded to obtain an immunoreactivity with G36-19 and that induced a poor definition of the bands. G36-19 recognized a comparable set of 33.5-, 36.5/40-, 49-, and 52-kD antigens but the reactivity was largely predominant over the 33.5-kD Ag, and the 36.5-kD and 40-kD Ag were not clearly resolved.

In the same experimental conditions, B17-21 was entirely unreactive.

Lastly, G36-19 and B17-21, assayed by immunoblotting from mono- and bi-dimensional gels of purified cytokeratins, showed no immunoreactivity to any of the 19 cytokeratins (not shown).

Antigen Expression in Cultured Keratinocytes After 3 weeks of culture, the normal keratinocytes from one subject (donor 1) produced a multilayered epidermal sheet lacking well organized SG and SC, whatever the culture conditions (Fig 8*a,c*). In all cases, IIF with G36-19 and B17-21 showed no labeling of the epidermal sheet (Fig 8*b,d*). In contrast, the keratinocytes from the second subject (donor 2) constituted completely differentiated epidermal sheets with a keratohyalin-granule–rich SG and a 5–6 corneocyte layer SC (Fig 8*e,g*). In the three non-standard "differentiating" conditions, all the morphologic signs of differentiation were increased; mainly, the number of corneocyte layers, the number and the size of keratohyalin granules, and the ratio of SC thickness to total epidermal sheet thickness. The condition that led to the most differentiated sheets was the absence of FCS in the culture medium (Fig 8*g*). To a lesser extent, the use of delipidized FCS also did and, lastly, the absence of EGF led to even fewer. In the four cases, IIF with both G36-19 and B17-21 produced microgranular diffuse cytoplasmic labeling of a single keratinocyte layer sandwiched between the SS and the SC, which were always entirely unreactive (Fig 8*f,h*). A clear correlation was found between the degree of morphologic differentiation of the four types of culture and the expression of the G36-19 and B17-21 Ag, which was the strongest in the cultures grown in the absence of FCS (Fig 8*h*).

DISCUSSION

In this paper we describe two Ag defined by the MoAb G36-19 and B17-21 that are expressed at a late stage of terminal differentiation in the human cornified epithelia. The Ag first located in the cytoplasm of lower SG keratinocytes acquired a pericellular location in the upper SG and so appeared to be secondarily associated with either the intercellular substance or cell periphery components. In most epidermal sites, the Ag ceased to be expressed from the lower SC, whereas they persisted up to the SC surface in plantar epidermis and hard palate epithelium, i.e., in non-stacked epithelia [53]. The Ag were also found to be expressed all along the three epithelial sheaths of the IRS in the HF with a similar distribution, first cytoplasmic microgranular then pericellular. This analogy of expression of G36-19 and B17-21 Ag in cornified squamous epithelia and in the cornified IRS epithelia can be related either to cross-reacting Ag or, more probably, to differentiation Ag shared by IRS and epidermis. Moreover, the persistence of G36-19 and B17-21 Ag all along the IRS up to its exfoliative area, as they persist up to the desquamating layer of plantar and hard palate SC, indicates a closer analogy between the differentiation of these epithelia.

The IIF analysis of corneocytes with G36-19 and B17-21 showed that the percentages of labeled cells differed according to their anatomical origin because they were much lower on corneocytes from arm and leg, where only 10 to 20% of the cells were labeled, than on corneocytes from sole and hard palate, where more than 90% of the cells were labeled. The cytomorphologic analysis of corneocytes showed that the two subpopulations of rough and smooth corneocytes, previously described as coming from lower and upper SC, respectively [51,52], largely vary in percentage from one epidermal site to another. Indeed, rough cells made up less than 20% of the non-palmoplantar corneocytes and about 90% of the plantar corneocytes. This result agrees with the regional differences in the surface pattern of superficial corneocytes described by scanning electron microscopy [54], and shows that in plantar SC the rough cells persist up to the uppermost horny layers. The concordance in percentages of rough corneocytes and corneocytes labeled by G36-19 and B17-21 suggests that the two populations are identical. This was clearly confirmed by the double examination by phase-contrast microscopy and IIF, which showed that only rough corneocytes express the G36-19 and B17-21 Ag. Thus, these Ag are borne by the rough corneocytes that are located throughout the whole SC in the non-stacked epidermis and only in the lower SC, in the more regularly stacked epidermis [53].

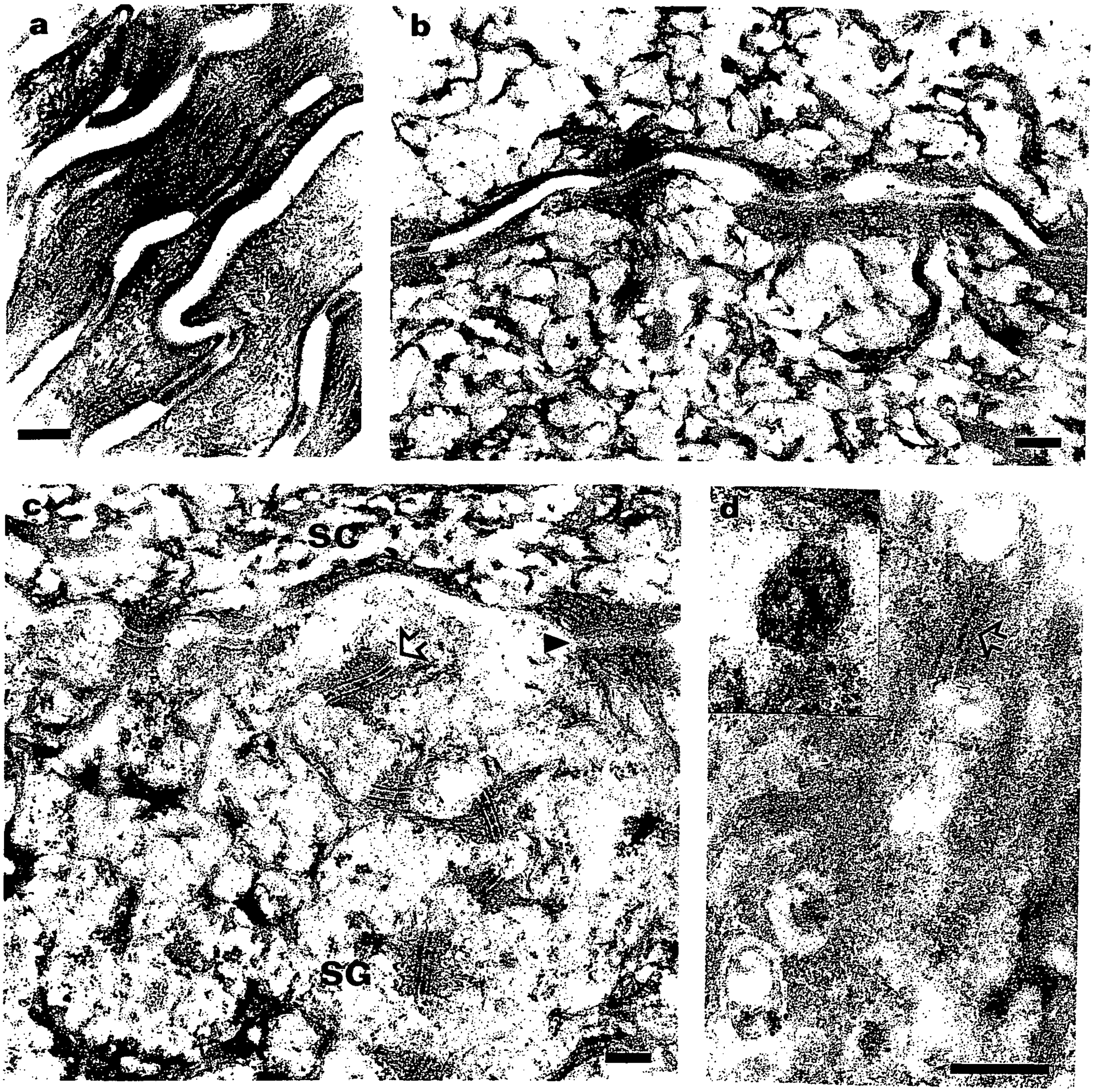
Whatever their anatomical origin, the labeled corneocytes showed the same labeling pattern with fluorescent spots regularly distributed over the cell surface. On corneocytes scraped from hard palate epithelium and plantar epidermis, the labeled spots were present in polygonal areas separated by unlabeled lines. In contrast, on the corneocytes from sites with stacked epidermis, the unlabeled lines were not observed. Therefore, the labeled spots were only present in the zones of vertical cell-to-cell contact, the unlabeled lines being related to the imprint of cell borders and intercellular spaces of the corneocytes located immediately above (or below) before corneocyte dissociation. The location, the density as well as the size of the spots, strongly suggested they correspond to a desmosome-like structure.

When we assayed G36-19 and B17-21 on CLE prepared from plantar SC, the labeling pattern observed resembled that of the corneocytes. The percentage of labeled CLE varied according to the method of preparation of the envelopes but, even after the most drastic purification, about 30% of the CLE still presented the Ag. Double examination by differential interference contrast microscopy and IIF showed that the G36-19 and B17-21 Ag are bound to the fragile CLE and completely absent from the rigid CLE. Recently, corneocytes from lower SC of non-palmo-plantar epidermis were shown to contain fragile immature CLE with an undulating surface, whereas corneocytes from upper SC were shown to contain rigid CLE with a smooth surface [15]. Because corneocytes and CLE undergo similar morphologic changes at the same locations during their ascension in the SC, it was obvious that rough corneocytes from low SC mainly contain fragile CLE, whereas smooth corneocytes from upper SC mainly contain rigid CLE. The exclusive location of G36-19 and B17-21 Ag on rough corneocytes and fragile CLE confirms the associations rough corneocyte-fragile CLE and smooth corneocyte-rigid CLE.

Immunoelectron microscopy confirmed that G36-19 and B17-21 decorate desmosomal structures. The Ag were first localized in the upper SS and in SG, in cytoplasmic vesicles of the size and structure of keratinosomes. Higher in the epidermis, the labeling was observed in the cores of SG desmosomes and in the amorphous intercellular cores of the modified desmosomes of the SC, i.e., the corneodesmosomes. The drastic ultrastructural modifications of desmosomes that occur at the SG/SC interface have been largely described [25-27]. The corneodesmosomes have been found to persist over the whole corneocyte surface up to the upper SC in non-stacked epidermis. In contrast, in stacked epidermis, they have been described with an overall cell-surface distribution only in the lower SC [26,55]. During cornification, a rarefaction of the corneodesmosomes from the lower to the upper SC was recently described by quantitative ultrastructural analysis [32] and the persisting corneodesmosomes were specifically localized in the periphery of the cells. Moreover, in the most regularly stacked epidermis, a complete disappearance of corneodesmosomes and appearance of a band structure running around the edges of the corneocytes, i.e., restricted to the overlapping areas of the uppermost corneocytes, and named "squamosome" have been reported [56]. With G36-19 and B17-21, we never observed a squamosome-like band even in non-palmo-plantar corneocytes. However, the transitory expression of the Ag that we found in various anatomical sites with well or irregularly stacked epidermis is concordant with the maturation of corneodesmosomes further processed from the corneocyte surface [32] and our immunoultrastructural results are compatible with this schema. In the same way, the persistence of Ag expression in the whole plantar SC and on scraped plantar corneocytes is closely concordant with the absence of corneodesmosome disappearance in the non-stacked plantar epidermis. The simultaneous persistence of corneodesmosomes and fragile CLE throughout the SC of plantar epidermis suggests that the transitory maturation state, characteristic of the lower SC in stacked epidermis, is maintained throughout the whole SC in the "physiologically hyperkeratotic" plantar epidermis.

The immunoultrastructural study of purified CLE allowed both their morphology to be described and the persistence of G36-19 and B17-21 Ag on their surface to be confirmed. Neither tonofilaments, nor desmosomal plates, nor amorphous corneodesmosomal cores were observed. The superficial electron-lucent layer on the CLE surface was the lipid portion previously described as a monolayer of hydroxyacylsphingosine molecules [19]. Its persistence on purified CLE agrees with its non-disulfide covalent anchoring to CLE components. The corneodesmosome areas, specifically labeled with G36-19 and B17-21, were distinguished by a more rigid appearance of the envelope and a better visualization of the clear lipid layer on their external side. The labeling was located on the top of micro-fibrillary projections, revealed after elimination of the corneodesmosome core, which possibly correspond to the extracellular portions of "transmembrane" CLE-linked molecules. The fuzzy network present on the internal cytoplasmic surface of CLE represents, at least partly, cytokeratin filaments linked to CLE, because it reacted with the anti-cytokeratin KL1 MoAb. It could also include other types of filaments joining the CLE to the fibrous intracellular matrix. The solid anchoring of these filaments along the entire CLE and the strong linking to the CLE of the corneodesmosomal molecules bearing the G36-19 and B17-21 Ag both persist even after drastic extraction under reducing and denaturing conditions. This suggests that the cell-to-cell cohesion and resistance to mechanical forces in SC may be related to a "fibrous matrix-CLE-corneodesmosome" tissue superstructure, replacing at the terminal differentiation stages the "cytokeratin intermediate filament network-desmosome" superstructure that fulfils this role in viable layers.

G36-19 recognized none of the 19 human cytokeratins. In contrast, in epidermal and SC soluble extracts, it defined a set of five Ag, with apparent molecular weights ranging from 33.5 to 52 kD. The Ag were entirely lysed by proteinase K and therefore were, at less partly, protein Ag. These proteins could either constitute a group of heteropolymers such as glycoproteins, all containing the same subunit bearing the G36-19 epitope, or they could represent polypeptides processed from a single precursor. Because of the greater immunoreactivity of the 52-kD Ag in blots from NP-40-TBS extracts of tape-stripped or whole epidermis, and, in contrast, the greater immunoreactivity of the 33.5-kD Ag in blots from SDS-2ME extracts of plantar SC, the 33.5-kD Ag could represent the ultimate SC form



of the processed protein and the 52-kD Ag its SS-SG precursor. The insolubility of the G36-19 Ag from plantar SC in NP-40-TBS suggests that during the transition from SG to SC, the NP-40-TBS-extractible precursors could be processed, linked to the CLE, and become unextractible. The weakness of the immunoreactivity on blots from SDS-2ME extracts, together with the persistence of immunoreactivity of fragile CLE even after drastic extraction with SDS-2ME, suggests that this linkage mainly corresponds to strong non-disulfide chemical bonds. Thus, the G36-19-defined epitope is borne by detergent-soluble proteins, probably synthesized late and located in keratinosome-like vesicles. The proteins are secondarily included in corneodesmosomes linked to fragile CLE and thus become weakly extractible. In upper SC of stacked epidermis, the proteins could be processed with the other corneodesmosome components and disappear from rigid CLE. When assayed by immunoblotting in the same conditions, B17-21 was always unreactive whatever the tissue substrates or the extraction methods used. The related epitope is thus probably conformational. However, all the histologic, cytologic, and ultrastructural data on B17-21, closely superimposable on those of G36-19, show that this MoAb is also specific for a corneodesmosomal component.

The numerous MoAb and antisera directed to squamous epithelia and producing pericellular labeling of keratinocytes that have been described to date were specific either for CLE-associated molecules such as CLE precursors [6-9,57,58] and transglutaminase [59] or for molecules involved in cell cohesion such as desmosome-specific

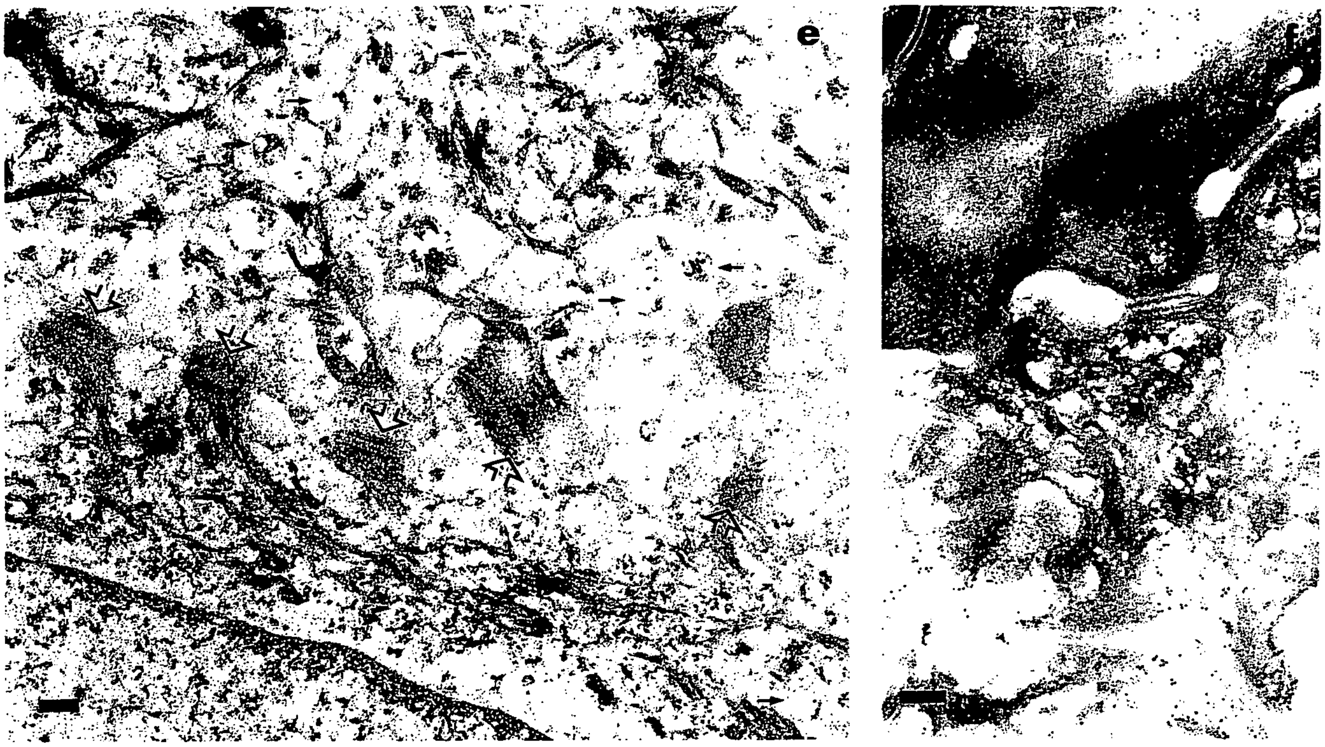


Figure 5. Post-embedding indirect immunogold labeling of abdominal epidermis with G36-19 *(a,b,c,d,e)* and, as a control, anti-cytokeratin KL1 MoAb *(f)*. In the stratum corneum *(a,b)*, intercellular cores of corneodesmosomes are the only structures to be labeled both between the upper *(a)* and the lower *(b)* corneocyte layers. At the interface (c) between the stratum granulosum (SG) and the stratum corneum (SC), both homogeneous intercellular bands of corneodesmosomes *(arrowhead)* and the characteristically structured cementing substance of typical desmosomes *(open arrow)* are labeled. Note the thick clear cross-linked envelope at the periphery of the first corneocytes (SC) and the dark keratohyalin granules in the SG keratinocytes. In the SG *(d)*, gold grains decorate cytoplasmic vesicles of size and shape compatible with keratinosomes, as well as regular desmosomes *(open arrows)*. The *insert* in *d* shows a labeled vesicle with some remnants of its lamellar structure. In keratinocytes from the lower SG and the upper SS *(e)*, small cytoplasmic vesicles *(little arrows)* are the only structures labeled with G36-19 MoAb; regular desmosomes *(open arrows)* remain negative at this level of epidermal differentiation. A positive control with anti-cytokeratin KL1 MoAb *(f)* shows no reactivity with the corneodesmosome in the SC (compare with *a*, *b*, and *c*). The Ab selectively binds to the cytokeratin filaments inside the keratinocytes. *Bars,* 200 nm.

components [60–65] or intercellular space glycoproteins [44,66]. The Ag defined by these Ab differed from the G36-19 and B17-21 Ag by their location in tissues and/or by their distribution on the cell surface, and always by their biochemical characteristics. Therefore, the G36-19 and B17-21 Ag appear to be new differentiation Ag.

The expression of G36-19 and B17-21 Ag by long-term cultured keratinocytes was found to be strongly related to the morphologic differentiation state reached by the epidermal sheet. When the epidermal sheets remained histologically non-cornified with one of the donors whatever the culture conditions, they were totally unreac-

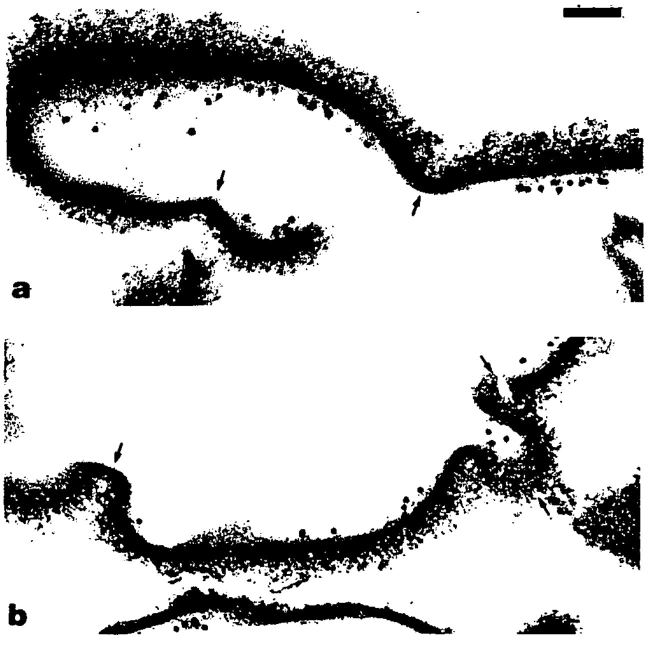


Figure 6. Pre-embedding indirect immunogold labeling of plantar cross-linked envelopes with G36-19 *(a)* and B17-21 *(b)* MoAb. The isolated cross-linked envelopes are exclusively labeled on their smoother extracellular side. Oblique section of the left part of the envelope in *(a)* shows the G36-19 reactivity with microfilamentous structures at the envelope surface. The gold grains bind to the exposed Ag epitopes that are separated from the fuzzy intracellular part by the electron-lucid hydroxyacylsphingosine layer and by a dense homogeneous band of the cross-linked proteins. Periodical immunogold-positive fragments of cross-linked envelope appear to be more rigid than the label-free portions *(arrows)*. Under the same experimental conditions, the labeling density with B17-21 MoAb *(b)* appears to be much lower than with G36-19 MoAb *(a)*. *Bar,* 100 nm.

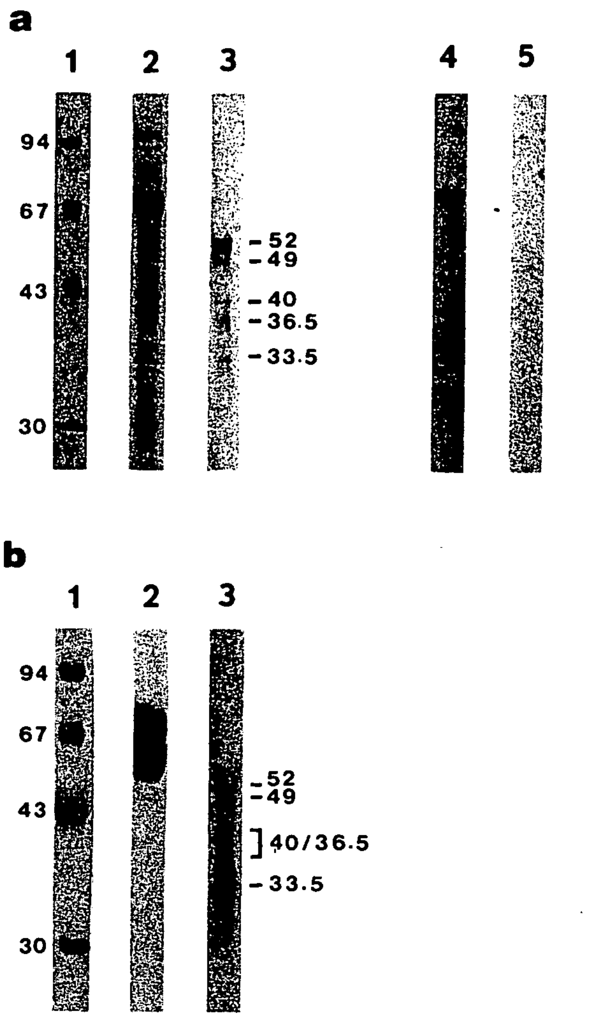


Figure 7. Immunoblotting reactivity of G36-19 MoAb on NP40-TBS *(a)* and SDS-2ME *(b)* soluble extracts from epidermis and from stratum corneum, separated by SDS-PAGE. *a*, extracts from whole breast epidermis *(lanes 2 and 3)* and from plantar stratum corneum *(lanes 4 and 5)* are stained by Coomassie blue *(lanes 2 and 4)* and immunoblotted with G36-19 MoAb *(lanes 3 and 5)*. G36-19 defines a set of five antigens from 33.5 to 52 kD in the epidermal extract and shows a major reactivity with the 52-kD band *(lane 3)*. On plantar stratum corneum extract it shows no reactivity *(lane 5)*. *Lane 1,* molecular weight standards: phosphorylase (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD). *b*, extract from plantar stratum corneum immunoblotted with EE21-06, a MoAb to cytokeratins number 1,2,9,10–11 *(lane 2)* and with G36-19 MoAb *(lane 3)*. G36-19 recognizes the same set of antigens from 33.5 to 52 kD and its reactivity is predominant over the 33.5-kD band. In the region 40/36.5 no bands are clearly resolved. *Lane 1,* molecular weight standards as above.

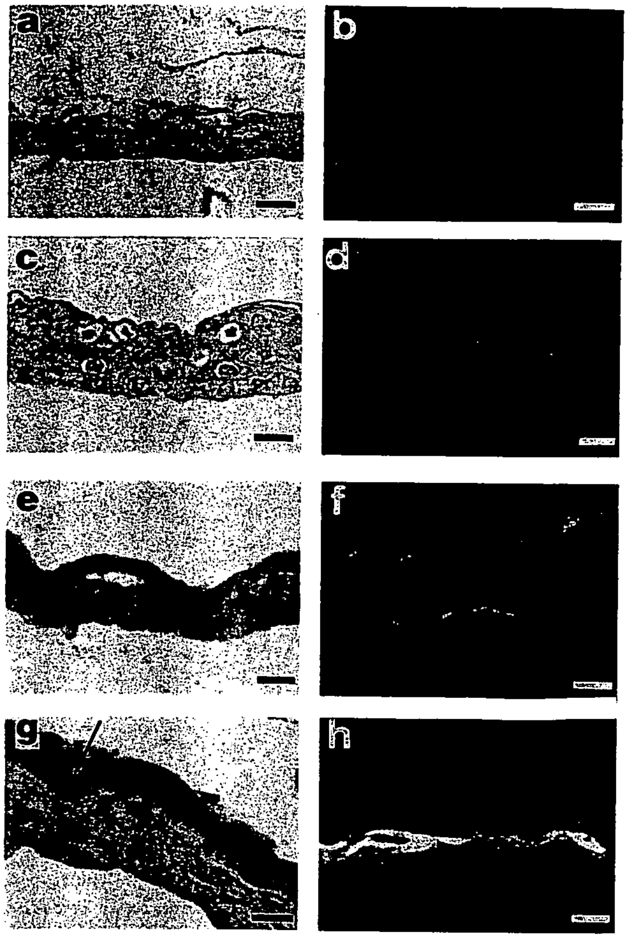


Figure 8. Morphology *(a,c,e,g)* and immunofluorescence with G36-19 MoAb *(b,d,f,h)* of sheets of cultured human keratinocytes from two subjects, donor 1 *(a,b,c,d)* and donor 2 *(e,f,g,h)*, cultured for 3 weeks under standard conditions *(a,b,e,f)*, with delipidized *(c,d)* and without *(g,h)* fetal calf serum (FCS). Keratinocytes from donor 1 constitute multilayered sheet without stratum granulosum or stratum corneum after culture in standard conditions *(a)* as well as with delipidized FCS *(c)*. In both cases there is no expression of the G36-19-defined Ag *(b,d)*. Keratinocytes from donor 2 constitute a multilayered sheet with a stratum granulosum showing numerous keratohyalin granules and a well-developed stratum corneum *(e,g)*. Keratohyalin granules are larger and more abundant *(arrow)* and cornified layers more numerous in cultures without FCS *(g)* than in standard cultures *(e)*. In both cases immunofluorescence with G36-19 *(f,h)* shows a labeling of stratum granulosum keratinocytes that is more intense and involves a larger number of cells in FCS-depleted cultures *(h)* than in standard cultures *(f)*. *Bar,* 15 *μ*m.

tive to MoAb. In contrast, with keratinocytes from the other donor, when the sheets underwent complete stratification with a multilayered SC, Ag synthesis did occur. Moreover, in the latter case, depending on the culture conditions, a higher degree of cornification led to higher Ag expression, which therefore appears as easily modulable in vitro. Thus, the desmosomal processing giving rise in vivo to corneodesmosomes probably also occurs in cornifying cultured epidermis.

Although in the brick wall model [24], the cohesion of the SC was thought to be mainly brought about by lipids, some recent results conflict with this hypothesis. It was demonstrated that proteins are involved in corneocyte cohesiveness and that proteases increase or even induce corneocyte dissociation [29,30]. Moreover, desmosomal proteins were recently thought to be involved in the bonds between plantar corneocytes because desmoglein I was shown to be proteolysed only in the uppermost desquamated corneocytes [67] and so is probably present in corneodesmosomes. Recently, using scanning and transmission electron microscopy, it was demonstrated that the increased number of corneodesmosomes at the surface of corneocytes, coming either from hyperkeratotic lesions or from hyperkeratotic SC induced in normal epidermis by repeated tape-stripping, was closely related with the difficulty or even the impossibility of further stripping the SC [33]. This gives a strong additional argument for a major role of corneodesmosomes in corneocyte cohesiveness. Regarding their ultrastructural location and their linkage to the CLE, it is highly probable that the Ag defined by G36-19 and B17-21 participate in the cohesive function of corneodesmosomes.

Thus, G36-19 and B17-21 Ag appear to be new differentiation markers of human cornified epithelia that can help to gain a better understanding of corneocyte cohesiveness. They can be efficient markers for studying the modulation of epidermis differentiation in vitro. Regarding their overexpression in the epidermis of healing wounds [68] and in hyperkeratotic cutaneous diseases such as psoriasis [69], the MoAb that define these Ag are relevant tools for studying distorsions of the cornification program and of desmosomal maturation in cutaneous pathology.

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REFERENCES

1. Franke WW, Schiller DL, Moll R, Winter S, Schmid E, Engelbrecht I, Denk H, Krepler R, Platzer E: Diversity of cytokeratins: differentiation-specific expression of cytokeratin polypeptides in epithelial cells and tissues. J Mol Biol 153:933-959, 1981
2. Skerrow D, Skerrow CJ: Tonofilament differentiation in human epidermis, isolation and polypeptide chain composition of keratinocyte subpopulations. Exp Cell Res 143:27-35, 1983
3. Eichner R, Bonitz P, Sun TT: Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression. J Cell Biol 98:1388-1396, 1984
4. Scott IR, Harding CR: Studies on the synthesis and degradation of a high molecular weight, histidine-rich phosphoprotein from mammalian epidermis. Biochim Biophys Acta 669:65-78, 1981
5. Kanitakis J, Ramirez-Bosca A, Réano A, Viac J, Roche P, Thivolet J: Filaggrin expression in normal and pathological skin. Virchows Archiv A Pathol Anat Histopathol 412:375-382, 1988
6. Zettergren JG, Peterson LL, Wuepper KD: Keratolinin: the soluble substrate of epidermal transglutaminase from human and bovine tissue. Proc Natl Acad Sci USA 81:238-242, 1984
7. Simon M, Green H: Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte. Cell 36:827-834, 1984
8. Simon M, Green H: Enzymatic cross-linking of involucrin and other proteins by keratinocyte particulates in vitro. Cell 40:677-683, 1985
9. Mehrel T, Hohl O, Rothnagel JA, Longley MA, Bundman D, Cheng C, Lichti U, Bisher ME, Steven AC, Steinert PM, Yuspa SH, Roop DR: Identification of a major keratinocyte cell envelope protein, Loricrin. Cell 61:1103-1112, 1990
10. Galvin C, Loomis C, Manabe M, Dhouailly D, Sun TT: The major pathways of keratinocyte differentiation as defined by keratin expression: an overview. Adv Dermatol 4:277-300, 1989
11. Holbrook KA: Biologic structure and function: Perspectives on morphologic approaches to the study of the granular layer keratinocyte. J Invest Dermatol 92:84S-104S, 1989
12. Jessen H: Two types of keratohyalin granules. J Ultrastruct Res 33:95-115, 1970
13. Hohl D: Cornified cell envelope. Dermatologica 180:201-211, 1990
14. Harding CR, Scott IR: Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. J Mol Biol 170:651-673, 1983
15. Michel S, Schmidt R, Shroot B, Reichert U: Morphological and biochemical characterization of the cornified envelopes from human epidermal keratinocytes of different origin. J Invest Dermatol 91:11-15, 1988
16. Scott IR, Harding CR, Barrett JG: Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. Biochim Biophys Acta 719:110-117, 1982
17. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of the human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31:11-24, 1982
18. Breitkreutz D, Bowden PE, Quinlan R, Herzmann E, Fusening NE: Proteolytic processing of prekeratins during terminal differentiation of mouse and human epidermis. J Submicrosc Cytol 16:23-25, 1984
19. Wertz PW, Downing DT: Covalently bound Ω-hydroxyacylsphingosine in the stratum corneum. Biochim Biophys Acta 917:108-111, 1987
20. Matoltsy AG, Parakkal PF: Membrane-coating granules of keratinizing epithelia. J Cell Biol 24:297-307, 1965
21. Madison KC, Swartzendruber DC, Wertz PW, Downing DT: Lamellar granule extrusion and stratum corneum intercellular lamellae in murine keratinocyte culture. J Invest Dermatol 90:110-116, 1988
22. Madison KC, Swartzendruber DC, Wertz PW, Downing DT: Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum. J Invest Dermatol 88:714-718, 1987
23. Shapiro LJ, Weiss R, Webster D, France JT: X-linked ichthyosis due to steroid-sulphatase deficiency. Lancet I: 70-72, 1978
24. Elias PM, Grayson S, Lampe MA, Williams ML, Brown BE: In: Marks R, Plewig G (eds.). The Intercorneocyte Space in Stratum Corneum. Springer-Verlag, Berlin-Heidelberg-New York, 1983, pp 53-67
25. Brody I: An electron-microscopic study of the junctional and regular desmosomes in normal human epidermis. Acta Derm Venereol 48:290-302, 1968
26. Allen TD, Potten CS: Desmosomal form, fate and function in mammalian epidermis. J Ultrastruct Res 51:94-105, 1975
27. White FH, Gohari K: Some aspects of desmosomal morphology during differentiation of hamster cheek pouch epithelium. J Submicrosc Cytol 16:407-422, 1984
28. Smith WP, Christensen MS, Nacht S, Gans EH: Effect of lipids on the aggregation and permeability of human stratum corneum. J Invest Dermatol 78:7-11, 1982
29. Bissett DL, McBride JF, Patrick LF: Role of protein and calcium in stratum corneum cell cohesion. Arch Dermatol Res 279:184-189, 1987
30. Lundström A, Egelrud T: Cell shedding from human plantar skin in vitro: evidence of its dependence on endogenous proteolysis. J Invest Dermatol 91:340-343, 1988
31. Brysk MM, Rajaraman S, Penn P, Barlow E, Bell T: Cohesive properties of terminally differentiated keratinocytes. Expl Cell Biol 57:60-66, 1989
32. Chapman SJ, Walsh A: Desmosomes, corneosomes and desquamation. An ultrastructural study of adult pig epidermis. Arch Dermatol Res 282:304-310, 1990
33. Shukuwa T, Udono M, Toyoshima H, Hirose R, Hori M, Yoshida H: Scanning electronmicroscopic and biochemical study of the corneocytes with villus-like projections (abstr). J Invest Dermatol 92:519A, 1989
34. Köhler GT, Milstein C: Continuous cultures of fused cells secreting antibodies of predefined specificity. Nature 256:459-497, 1975
35. Serre G, Vincent C, Viraben R, Soleilhavoup JP: Natural IgM and IgG autoantibodies to epidermal keratins in normal human sera. I. ELISA titration, immunofluorescence study. J Invest Dermatol 88:21-27, 1987
36. Guilbert B, Dighiero G, Avrameas S: Naturally occurring antibodies against nine common antigens in human sera. I. Detection, isolation, and characterization. J Immunol 128:2779-2787, 1982
37. Viac J, Réano A, Brochier J, Staquet MJ, Thivolet J: Reactivity pattern of a monoclonal antikeratin antibody (KL1). J Invest Dermatol 81:351-354, 1983
38. Haftek M, Staquet MJ, Viac J, Schmitt D, Thivolet J: Immunogold labeling of keratin filaments in normal human epidermal cells with two anti-keratin monoclonal antibodies. J Histochem Cytochem 34:613-618, 1986
39. Haftek M, Chignol MC, Thivolet J: Quantitative studies of keratin expression with the post-embedding immunogold labeling method. J Histochem Cytochem 37:735-741, 1989
40. Yonish-Rouach E, Schmitt D, Viac J, Knowles R, Cordier G, Brochier J: Monoclonal anti-thymic cell antibodies detecting epidermal cells. Thymus 6:67-78, 1984

41. Mansbridge JN, Knapp AM: Changes in keratinocyte maturation during wound healing. J Invest Dermatol 89:253-263, 1987
42. Dale BA, Holbrook KA, Kimball JR, Hoff M, Sun TT: Expression of epidermal keratins and filaggrin during human fetal skin development. J Cell Biol 101:1257-1269, 1985
43. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970
44. Brysk MM, Rajaraman S, Penn P, Chen SJ; Endogenous lectin from terminally differentiated epidermal cells. Differentiation 32:230-237, 1986
45. Achtstätter T, Hatzfeld M, Quinlan RA, Parmelee DC, Franke WW: Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. Meth Enzymol 134:335-371, 1986
46. O'Farrell PZ, Goodman HM, O'Farrell PH: High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133-1142, 1977
47. Franke WW, Denk H, Kalt R, Schmidt E: Biochemical and immunological identification of cytokeratin proteins present in hepatocytes of mammalian liver tissue. Exp Cell Res 131:299-318, 1981
48. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350-4354, 1979
49. Serre G, Conter A, Ouhayoun JP, Vincent C, Bena C, Mils V, Dueymes M, Sommé G, Soleilhavoup JP: EE21-06 a mouse monoclonal antibody to a sequential epitope common to the keratin polypeptides specifically expressed in the human keratinized squamous epithelia. In: Rousset BAS (ed.). Structure and Functions of the Cytoskeleton. John Libbey Eurotex/Editions INSERM, London-Paris, 1988, p 524
50. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331-344, 1975
51. King CS, Barton SP, Nicholls S, Marks R: The changes in properties of the stratum corneum as a function of depth. Br J Dermatol 100:165-172, 1979
52. Barton SP, King CS, Marks R, Nicholls S: A technique for studying the structural detail of isolated human corneocytes. Br J Dermatol 102:63-73, 1980
53. Mackenzie IC, Zimmerman K, Peterson L: The pattern of cellular organization of human epidermis. J Invest Dermatol 76:459-461, 1981
54. Heilmann BB, Ryckmanns F, Plewig G: Scanning electron microscopy of human corneocytes. In: Stratum Corneum. Marks R, Plewig G (ed.). Springer-Verlag, Berlin-Heidelberg-New York, 1983, pp 186-189
55. Allen TD, Potten CS: Ultrastructural site variations in mouse epidermal organization. J Cell Sci 21:341-359, 1976
56. Allen TD, Potten CS: Fine-structural identification and organization of the epidermal proliferative unit. J Cell Sci 15:291-319, 1974
57. Ma ASP, Sun TT: Differentiation-dependent changes in the solubility of a 195-kD protein in human epidermal keratinocytes. J Cell Biol 103:41-48, 1986
58. Baden HP, Kubilus J, Phillips SB: Characterization of monoclonal antibodies generated to the cornified envelope of human cultured keratinocytes. J Invest Dermatol 89:454-459, 1987
59. Michel S, Démarchez M: Localization and in vivo activity of epidermal transglutaminase. J Invest Dermatol 90:472-474, 1988
60. Cohen SM, Gorbsky G, Steinberg MS: Immunochemical characterization of related families of glycoproteins in desmosomes. J Biol Chem 258:2621-2627, 1983
61. Osborn M, Weber K: A monoclonal antibody recognizing desmosomes: use in human pathology. J Invest Dermatol 85:385-388, 1985
62. Franke WW, Cowin P, Schmelz M, Kapprell HP: The desmosomal plaque and the cytoskeleton. In Bock G, Clark S (eds.) Junctional Complexes of Epithelial Cells. Wiley, Chichester, 1987, pp 26-48
63. Franke WW, Schmid E, Grund C, Müller H, Engelbrecht I, Moll R, Stadler J, Jarasch ED: Antibodies to high molecular weight polypeptides of desmosomes: specific localization of a class of junctional proteins in cells and tissues. Differentiation 20:217-241, 1981
64. Parrish EP, Garrod DR, Mattey DL, Hand L, Steart PV, Weller RO: Mouse antisera specific for desmosomal adhesion molecules of suprabasal skin cells, meninges, and meningioma. Proc Natl Acad Sci USA 83:2657-2661, 1986
65. Schmelz M, Duden R, Cowin P, Franke WW: A constitutive transmembrane glycoprotein of Mr 165000 (desmoglein) in epidermal and non-epidermal desmosomes. I. Biochemical identification of the polypeptide. Eur J Cell Biol 42:177-183, 1986
66. King CS, Dykes PJ, Marks R: Preparation and characterization of the nonionic detergent-soluble proteins of human stratum corneum. J Invest Dermatol 79:297-302, 1982
67. Egelrud T, Lundström, A: Immunochemical analysis of the distribution of the desmosomal protein desmoglein I in different layers of plantar epidermis. Acta Dermato Venereo 69:470-476, 1989
68. Serre G, Rigal C, Mils V, Pieraggi MT, Sommé G, Soleilhavoup JP, Bouissou H: Neo-epidermis differentiation during cutaneous wound healing in the pig, studied with 2 monoclonal antibodies specific for the cornified squamous epithelia (abstr). J Invest Dermatol 92:146A, 1989
69. Haftek M, Serre G, Carrier V, Mils V, Roche P, Thivolet J: Studies on the distribution of the corneodesmosome antigen G36-19 in various states of pathological keratinization (abstr). J Invest Dermatol 94:397A, 1990

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